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The Polerovirus Minor Capsid Protein Determines Vector Specificity and Intestinal Tropism in the Aphid

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Aphid transmission of poleroviruses is highly specific, but the viral determinants governing this specificity are unknown. We used a gene exchange strategy between two poleroviruses with different vectors, *Beet western yellows virus* (BWYV) and *Cucurbit aphid-borne yellows virus* (CABYV), to analyze the role of the major and minor capsid proteins in vector specificity. Virus recombinants obtained by exchanging the sequence of the readthrough domain (RTD) between the two viruses replicated in plant protoplasts and in whole plants. The hybrid readthrough protein of chimeric viruses was incorporated into virions. Aphid transmission experiments using infected plants or purified virions revealed that vector specificity is driven by the nature of the RTD. BWYV and CABYV have specific intestinal sites in the vectors for endocytosis: the midgut for BWYV and both midgut and hindgut for CABYV. Localization of hybrid virions in aphids by transmission electron microscopy revealed that gut tropism is also determined by the viral origin of the RTD.

The genera *Polerovirus*, *Luteovirus*, and *Enamovirus* constitute the *Luteoviridae* family (luteovirids). Luteovirids are restricted to the phloem tissue of host plants and are strictly transmitted by aphids in a persistent, circulative, and nonpropagative manner (17, 20). Virions, acquired by aphids while feeding from sieve tubes of an infected plant, are transported through the gut epithelium and released in the hemolymph. In this compartment, virions interact with symbionin (of endosymbiont origin), which may protect them from the immune system and/or modify structural properties of virions (42). Virus particles in the hemolymph can be taken up by accessory salivary gland (ASG) epithelial cells from which they are released via the salivary duct during feeding.

Transmission electron microscope (TEM) observations indicate that transport of virions through the gut and ASG epithelia occurs by receptor-mediated endocytosis/exocytosis. Passage across the gut wall can take place at two sites, the posterior midgut and the hindgut. *Barley yellow dwarf virus*-MAV (*Luteovirus*) (16), *Cereal yellow dwarf virus* (*Polerovirus*) (17), and *Soybean dwarf virus* (unassigned member of the *Luteoviridae*) (19) are internalized at the hindgut, while the posterior midgut is used by two poleroviruses, *Beet western yellows virus* (BWYV) (37) and *Potato leafroll virus* (14). *Cucurbit aphid-borne yellows virus* (CABYV) (*Polerovirus*) is unique among studied luteovirids in that its virions are taken up at both sites (38).

Luteovirus transmission is highly specific (21), but the molecular mechanisms controlling specificity are unknown. One hypothesis is that specificity is mediated by interaction between motifs on the virion and receptors at the epithelial cell plasmalemma during endocytosis. Experiments with different combinations of luteovirids and vector or nonvector aphid species indicate that the basal plasmalemma of ASG epithelial cells is an important site for such differential interactions, although the ASG basal lamina can also act as a virus-specific filter for some virus-aphid combinations (18, 19, 34). The gut epithelium, on the other hand, appears to be a relatively permissive barrier, since most of the luteovirids studied can be acquired in the hemolymph of nonvector aphid species (16, 19). Symbionin, which is present in the hemolymph of both vector and nonvector aphid species, is unlikely to account for vector specificity (42).

Luteovirids form 25-nm-diameter icosahedrical particles containing an RNA genome of ca. 6 kb. The capsid is composed of two structural proteins, the ca. 21-kDa major coat protein (CP) encoded by open reading frame (ORF) 3, and a minor component, the ca. 75-kDa readthrough (RT) protein, which is a fusion of the CP and the readthrough domain (RTD) (encoded by ORF5) (Fig. 1). The RTD is exposed on the surface of the particle (5) and is required for efficient virus movement in infected plants (5, 9, 30). Mutagenesis of full-length infectious clones and biological studies have mapped amino acid sequences on the CP and the RTD which are important for the transmission process (3–5, 8, 9, 25), but such studies do not permit identification of motifs governing vector specificity. In this paper, we have produced infectious recombinant viruses in which the RTD sequences of BWYV and CABYV have been exchanged. The hybrid virions were then used in transmission studies with aphid species displaying differential transmission efficiency for BWYV and CABYV. The viral hybrids were also used to study the role of the RTD in specifying intestinal tissue tropism of BWYV and CABYV.

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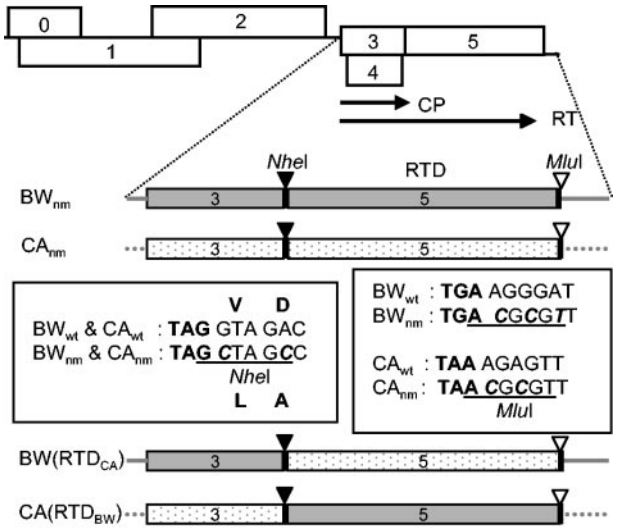

FIG. 1. Schematic representation of ORFs 3 and 5 of mutated and recombinant viruses. The genetic organization of polerovirus genome encoding the five ORFs is presented. The encoded major (CP) and minor (RT) coat proteins are indicated by arrows. Positions of the unique NheI (▼) and MluI (▽) restriction sites introduced up- and downstream of the RTD gene (ORF5) to obtain the recombinant viruses are indicated. Intermediate mutated viruses obtained during the cloning procedure (BWn and CAn) carry only the nucleotide changes creating the NheI site downstream of the ORF3 sequence. Nucleotide changes introduced to create the two restriction sites are shown in the boxes in italic letters with reference to the wild-type sequences of CABYV and BWYV. The amino acid replacement (VD to LA) is indicated.

MATERIALS AND METHODS

BWYV and CABYV RTD recombinants. Recombinant BWYV and CABYV cDNAs were obtained by exchanging the RTD nucleotide sequences between full-length infectious clones of BWYV (44) and CABYV (36). PCR-based mutagenesis was used to introduce unique restriction sites into the full-length cDNA clones of the two viruses. An NheI site was introduced immediately downstream of the CP stop codon of each cDNA to produce pBWn and pCAn. An MluI site was then introduced into pCAn and pBWn just downstream of the RTD (ORF5) stop codon, leading to pBWnm and pCAnm, respectively (Fig. 1). BW(RTD_{CA}) was obtained by insertion of the 1,404-bp NheI-MluI RTD fragment from pCAnm into NheI-MluI-digested pBWnm, and the reverse recombinant CA(RTD_{BW}) was obtained by introducing the 1,404-bp NheI/MluI fragment from pBWnm into NheI-MluI-digested pCAnm. Constructs for agroinfection were made by replacing the wild-type BWYV or CABYV cDNA sequences in the binary plasmid $pBinBW_0$ (5) or pBin35SCA-WT (36). The resulting plasmids were introduced into *Agrobacterium tumefaciens* C58C1 (23) for agroinoculation.

Infection of protoplasts and plants. Full-length RNA transcripts were produced and inoculated into *Chenopodium quinoa* protoplasts as described previously (8). *A. tumefaciens* harboring binary plasmids was grown to an optical density (OD) at 600 nm of 1 and agroinfiltrated (13) into *Montia perfoliata*, *Nicotiana clevelandii*, and *Cucumis sativus* as described previously. Infected plants were identified 4 to 5 weeks postinoculation (p.i.) by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with a rabbit polyclonal antiserum raised against each virus (8, 26).

Total RNA was isolated from systemically infected leaves of plants which have been agroinfected or aphid inoculated with BWnm or CAnm. Reverse transcription followed by PCR using specific primers flanking the RTD sequence was used to characterize the sequence encoding the RTD in the progeny virus as described previously (4).

Nucleotide sequences between positions 4098 and 5655 in the BWYV genome were amplified and cloned, and inserts from randomly selected clones were sequenced. For CABYV, two overlapping fragments were amplified (nucleotides [nt] 4098 to 4986 and 4901 to 5655), cloned, and then sequenced. In both cases, the amplified fragments covered the entire RTD sequence, including the mutations creating the novel NheI and MluI sites as well as the 3′ noncoding region.

Aphid transmission experiments. Virus-free colonies of *Myzus persicae*, *Aphis gossypii*, and *Macrosiphum euphorbiae* were reared on caged pepper (*Capsicum annuum*), cucumber (*Cucumis sativus*), and eggplant (*Solanum melongena*) seedlings, respectively, at 20°C with a 16-h photoperiod. Aphid transmission experiments used either purified virus or detached leaves from agroinfected plants as a source of inoculum (8). Purified suspensions of virus were obtained from agroinfected *M. perfoliata* as previously described (43) and offered to aphids in a solution of 0.1 M sodium citrate, pH 6, and 20% sucrose. Third- and fourth-instar nymphs or adults were given a 24-h acquisition access period (AAP) before being transferred to *M. perfoliata* test plants. Infection of test plants was monitored by DAS-ELISA 4 weeks later. Microinjection of purified virions into the hemocoel was performed as described previously (8). The AAP was extended to 72 to 96 h when aphids were destined for ultrastructural observations (37).

Western and Northern blotting. Viral structural proteins in total protein extracts of infected protoplasts or plants were detected by Western blotting using antisera specific for BWYV CP or RTD (4). The polyclonal antiserum used for ELISA was also used for detection of the CABYV CP. An antiserum raised against a glutathione-*S*-transferase fusion protein containing the C-terminal part of the CABYV RTD (nt 4882 to 5508) was used to detect the CABYV RTD. Detection was performed with enhanced chemiluminescence Western blotting kit (Bio-Rad, France). Viral RNAs extracted from infected plants or protoplasts (3) were detected by Northern blot using either a [^{32}P]UTP-labeled probe or a digoxigenin-labeled probe (Boehringer, Germany). The probes were complementary to the noncoding 3′-terminal 196 and 201 nt of the BWYV and CABYV genomes, respectively (36, 44).

RESULTS

Infectivity of recombinant viruses in protoplasts. To study the role of the CP and the RTD of BWYV and CABYV in determining vector specificity, we constructed the hybrid viruses BW(RTD_{CA}) and CA(RTD_{BW}) (Fig. 1) by exchanging the ORF5 sequence between BWYV and CABYV in full-length infectious cDNA clones. To facilitate the exchange, unique NheI and MluI sites were introduced upstream and downstream of the ORF5 sequence in each full-length clone to produce the mutant viruses BWnm and CAnm (Fig. 1). Two intermediate clones (CAn and BWn) containing only the NheI site were also obtained during the cloning procedure. Creation of the NheI site at the beginning of ORF5 required modification of two nucleotides in the BWYV and CABYV sequences (which are identical in this region), resulting in replacement of the first two amino acids of the wild-type RTD sequence of each virus (VD) by LA (Fig. 1). Introduction of the MluI site just downstream of the ORF5 termination codon required three nucleotide substitutions in the BWYV sequence and two nucleotide substitutions in that of CABYV (Fig. 1). Viral transcripts corresponding to BWn, BWnm, CAn, CAnm, BW(RTD_{CA}), and CA(RTD_{BW}) were inoculated to *C. quinoa* protoplasts. The BWn and CAn transcripts directed synthesis of viral genomic and subgenomic RNA in amounts similar to those observed for BWwt and CAwt (Fig. 2). However, a significant reduction of progeny viral RNA accumulation was observed for BWnm, CAnm, BW(RTD_{CA}), and CA(RTD_{BW}) (Fig. 2), suggesting that the nucleotide substitutions introduced to create the MluI sites downstream of the RTD sequence diminished viral RNA replication rates by altering a *cis*-acting RNA sequence or structure involved in initiation of negative-strand RNA synthesis.

Accumulation of recombinant viruses in agroinfected plants. Full-length viral cDNA containing the mutated or chimeric sequences was moved into the binary vector pBin19 under control of the *Cauliflower mosaic virus* 35S promoter, and the

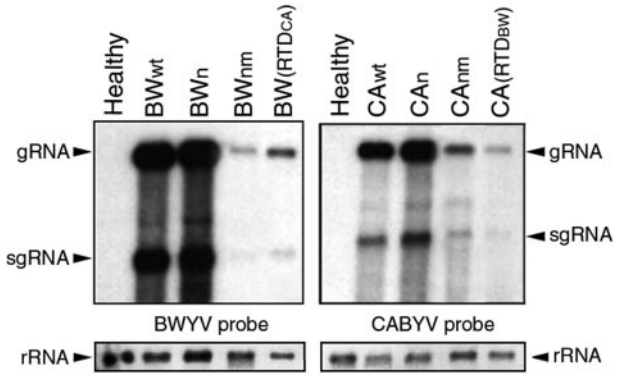

FIG. 2. Accumulation in protoplasts of RNA of mutated and recombinant viruses. Shown is Northern blot analysis of total RNA extracted from protoplasts inoculated with transcripts corresponding to the different mutated and chimeric viruses. RNA was extracted from mock-inoculated protoplasts (Healthy). Positions of genomic (g) and subgenomic (sg) RNAs are indicated. ^{32}P-labeled probes specific for each virus (BWYV or CABYV probe) were used. rRNAs were stained with ethidium bromide.

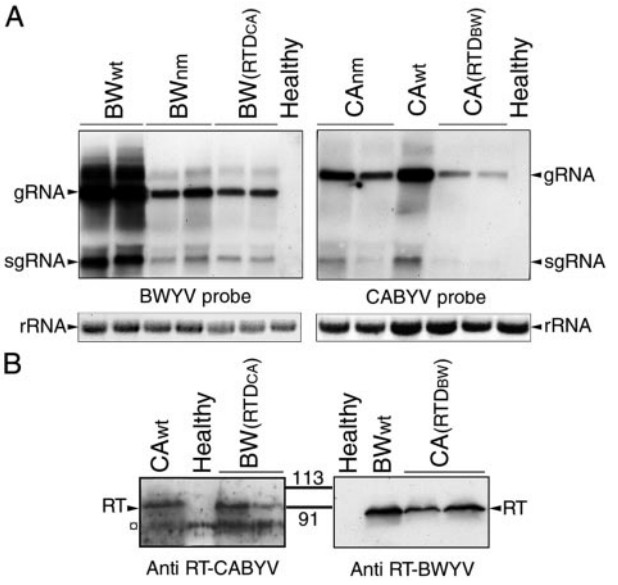

FIG. 3. Accumulation of recombinant viruses in agroinfected *M. perfoliata*. (A) Northern blot analysis of total RNA extracted 5 weeks p.i. RNA was extracted from noninoculated plants (Healthy). Positions of genomic (g) and subgenomic (sg) RNAs are indicated. Virus-specific digoxigenin-labeled probes were used. rRNAs were stained with ethidium bromide. (B) Immunodetection of RT proteins. Protein extracts were prepared from ELISA-positive plants 5 weeks p.i. and from noninoculated (Healthy) plants. The volume of protein extract from BW(RTD_{CA})- and CA(RTD_{BW})-infected plants loaded onto the gel was doubled compared to extracts from CAwt- and BWwt-inoculated plants. Membranes were probed with the indicated specific antiserum. The square to the left indicates a background band due to cross-reaction of anti-RT-CABYV antibodies with an unidentified host protein. Positions of 91- and 113-kDa marker proteins are indicated.

resulting binary plasmids were agroinoculated to either (i) *M. perfoliata*, a common host for BWYV and CABYV; (ii) *N. clevelandii*, a BWYV-specific host; or (iii) *C. sativus*, a CABYV-specific host (26). Virus titers in noninoculated upper leaves were assayed by DAS-ELISA 4 weeks postinoculation using a polyclonal antiserum directed against CABYV for detection of CAwt and its derivatives [CAnm and CA(RTD_{BW})] and a BWYV polyclonal antiserum for detection of BWwt and its derivatives [BWnm and BW(RTD_{CA})]. All constructs were infectious in *M. perfoliata* (Table 1), illustrating that the chimeric RT proteins can drive virus movement in whole plants. Consistent with the accumulation rates observed in infected protoplasts, progeny genomic and subgenomic RNA of BWnm, CAnm, and the hybrid viruses BW(RTD_{CA}) and CA(RTD_{BW}) accumulated at a reduced level compared to those of BWwt and CAwt (Fig. 3A).

The stability of the engineered mutations (NheI and MluI sites) in viral progeny of BWnm and CAnm in *M. perfoliata* was investigated by reverse transcription-PCR. As observed previously (4), viral progeny in plants infected with the constructs accumulated a low level of secondary site mutations all along the RTD sequence, but none of the mutations observed affected the original mutations introduced (Fig. 4). The mutation rate was higher in the CAnm viral progeny than the BWnm progeny, but with one exception, no mutation hot spots were detected in the RTD sequence. The exception was a high

TABLE 1. ELISA detection of mutated and recombinant viruses in agroinfected plants[a]

Mutant	Plant species[d]					
	M. perfoliata		*N. clevelandii*		*C. sativus*	
	No. inf./No. inoc.[b] (%)	ELISA OD[c]	No. inf./No. inoc. (%)	ELISA OD	No. inf./No. inoc. (%)	ELISA OD
BWwt	11/16 (69)	1.87 ± 0.40	18/19 (95)	1.63 ± 0.27	0/14	0.12 ± 0.01
BWnm	30/46 (65)	1.68 ± 0.35	38/43 (88)	1.42 ± 0.19	0/18	0.11 ± 0.01
BW(RTD_{CA})	13/26 (50)	1.51 ± 0.40	25/34 (74)	0.57 ± 0.17	0/20	0.11 ± 0.01
Noninoc.	0/7	0.13 ± 0.02	0/7	0.12 ± 0.01	0/2	0.11 ± 0.00
CAwt	22/32 (69)	2.13 ± 0.10	0/13	0.12 ± 0.01	14/27 (52)	1.44 ± 0.50
CAnm	16/44 (36)	1.70 ± 0.36			6/9 (67)	0.73 ± 0.51
CA(RTD_{BW})	26/66 (39)	1.29 ± 0.53	27/44 (61)	1.02 ± 0.53	9/15 (60)	0.27 ± 0.03
Noninoc.	0/10	0.13 ± 0.01	0/5	0.12 ± 0.02	0/4	0.12 ± 0.01

[a] Plant extracts were tested by DAS-ELISA 4 weeks p.i. with an antiserum raised against BWYV for detection of BWwt, BWnm, and BW(RTD_{CA}) or against CABYV for detection of CAwt, CAnm, and CA(RTD_{BW}).
[b] Number of plants infected/number of plants agroinoculated. In parentheses, the percentage of infected plants is shown.
[c] Mean absorbance ± standard error of infected or noninoculated (noninoc.) plants at 405 nm after 2 h of substrate incubation.
[d] Results from two to four independent experiments were combined.

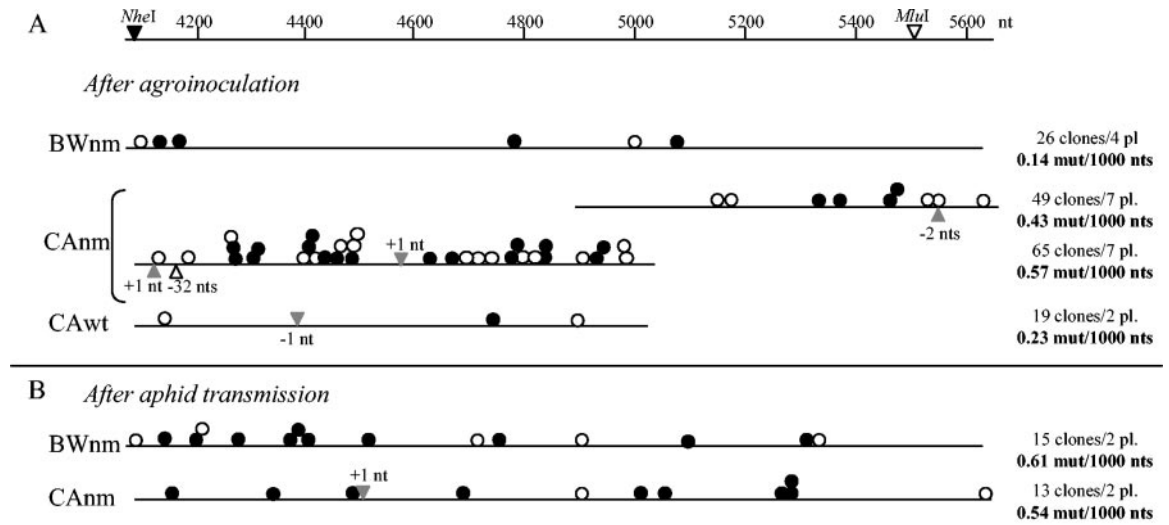

FIG. 4. Distribution of second-site mutations detected in viral RNA progeny of CAnm and BWnm. Analysis of viral progeny (A) following agroinfection of *M. perfoliata* with BWnm, CAnm, or CAwt or (B) after aphid transmission to *M. perfoliata* is shown. Positions of mutations introduced to create NheI and MluI sites are indicated. For each virus, the horizontal line represents the sequenced RTD domain. At the right, the number of clones obtained from different plants that were analyzed and the number of mutations found per 1,000 nt sequenced are indicated. Open circles denote silent mutations, and filled circles represent mutations which provoke an amino acid change. Small triangles refer to insertions or deletions of nucleotides.

frequency of mutation (58%) present in the 13-residue C tract located downstream of the CABYV CP stop codon (Table 2). These mutations corresponded to the addition or deletion of one or more C residues and would introduce a frameshift in the genome sequence of viral progeny. Similar modifications were also observed in reverse transcription-PCR products obtained from CAwt viral progeny as well as after PCR amplification of the corresponding cloned DNA fragment (Table 2). Therefore, the length heterogeneity observed in the C tract of CAnm most probably resulted from "slippage" of the *Taq* polymerase on this homopolymeric sequence during PCR amplification, as was previously shown to occur on a 26-nt poly(A) stretch (10). However, the possibility that some of the observed mutations arose from errors introduced by the viral polymerase during virus replication cannot be strictly eliminated. We conclude that, with the exception of a low level of mutations which appear to be scattered randomly along the RTD sequence and which presumably arise from errors during the replication process, the sequence of the progeny viral RNA closely resembles that of the input inoculum.

TABLE 2. Number of C residues in the C tract of reverse transcription-PCR products obtained from viral progeny in agroinfected *M. perfoliata*

Viral progeny	No. of mutations in C tract of length (residues)						% wt sequence[c]
	10	11	12	13	14	15	
CAnm progeny	1	3	20	27	12	2	42
CAwt progeny[a]		2	5	11	1		58
CAwt DNA[b]		1	2	5			62

[a] The CAwt sequence contains 13 C residues.
[b] PCR amplification was performed on a cloned DNA fragment of CAwt containing the C tract.
[c] wt, wild type.

In *N. clevelandii*, agroinoculation of BWwt and BWnm readily induced infection, but CAwt failed to infect this known CABYV nonhost (Table 1). Interestingly, both CA(RTD_{BW}) and BW(RTD_{CA}) were infectious to *N. clevelandii* (Table 1), although virus titers in infected leaves were somewhat lower for BW(RTD_{CA}) than for BWwt or BWnm. As expected, *C. sativus* was infected by CAwt and CAnm but not by BWwt and BWnm (Table 1). Of the two recombinant viruses, BW(RTD_{CA}) did not infect *C. sativus*, and only a very weak infection (reflected by low ELISA values for infected plants) was observed for CA(RTD_{BW}) (Table 1).

Western blot analysis was used to characterize the viral structural polypeptides in agroinfected *M. perfoliata*. The full-length RT protein of BWYV and other luteovirids has sodium dodecyl sulfate-polyacrylamide gel electrophoresis mobility of a ca. 90-kDa protein (2, 5). RTD-specific antibodies specifically detected a polypeptide of this mobility in crude protein extracts from plants infected with BWwt, CAwt, BW(RTD_{CA}), or CA(RTD_{BW}) (Fig. 3B). Note that more extract was loaded for the plants infected with the recombinant viruses to obtain comparable amounts of the chimeric and wild-type RT proteins in all the extracts.

The protein contents of purified virions were also investigated by Western blot. As previously observed, luteovirid RT protein in purified virus preparations exists as a C-terminally-truncated form, called RT*, with an apparent molecular mass of ~67 kDa (6). Figure 5 shows that RT* is also present in the hybrid virions purified from infected *M. perfoliata*, although in much lower amounts than in BWwt and CAwt virions. These results do not permit differentiation between a uniformly reduced incorporation of the chimeric RT protein into virions and the existence of a heterogeneous population of virions containing more or less RT protein. Possibly, the hybrid nature of the chimeric RT proteins interferes with their efficient in-

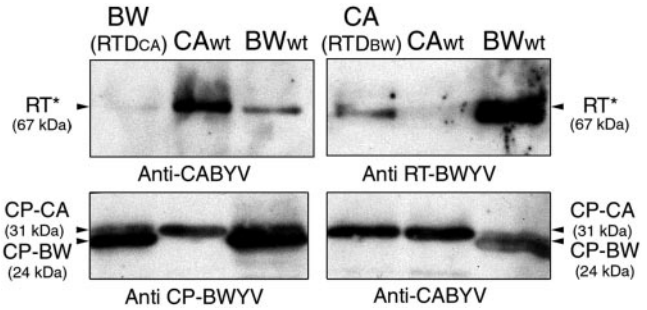

FIG. 5. Immunodetection of capsid proteins in virus purified from *M. perfoliata*. The upper and lower parts of the left- and right-hand panels are each from one single membrane split into two before incubation with the indicated antibodies. The upper part of the membrane was probed with an anti-CABYV antiserum (upper left panel) or with antibodies raised against the BWYV RTD (anti-RT-BWYV, upper right panel). The position of C-terminally-truncated RT protein (RT*) is indicated. The lower part of the membrane was incubated with either the anti-CABYV antiserum (lower right panel) or antiserum specific for BWYV CP (anti-CP-BWYV, lower left panel). The positions of CABYV and BWYV CP are indicated with apparent molecular masses in brackets.

corporation into virus particles. Alternatively, the hybrid RT proteins may be more readily lost from virions during the purification procedure. In view of the extensive sequence conservation among polerovirus CPs (27), it was not surprising that the antisera used to detect the BWYV and CABYV CPs cross-reacted with the noncognate CP as well (Fig. 5, lower panel). However, the difference in mobility between BWYV and CABYV CPs allowed the two species to be discriminated on the Western blots (Fig. 5, lower panel). Shared CP epitopes can also account for recognition of the BWYV RT protein by the CABYV-specific antibodies (Fig. 5, upper left panel).

Vector specificity of the virus recombinants. A first series of transmission experiments used fully expanded leaves of agroinfected *M. perfoliata* or *N. clevelandii* (4 to 6 weeks p.i.) as a virus source. The transmissibility of BW(RTD_{CA}) and CA(RTD_{BW}) was assayed with two aphid species which can discriminate between BWYV and CABYV. *A. gossypii* transmits CABYV but not BWYV (26), and *M. euphorbiae* displays differential transmission efficiency for the two viruses under nonoptimal experimental conditions (see below). *M. persicae*, a vector of both BWYV and CABYV (26), was included in the transmission experiments as an internal control.

Using infected *M. perfoliata* as a virus source and *M. persicae* as a vector, BWwt, BWnm, CAwt, and CAnm were readily transmitted to *M. perfoliata* (Table 3). Viral progeny in BWnm and CAnm aphid-inoculated plants exhibited several second-site mutations randomly distributed along the RTD sequence (Fig. 4). Thus, aphid transmission does not seem to act as a bottleneck reducing heterogeneity in the viral population found in agroinoculated plants. None of the changes observed in the RTD sequence affected the original mutations introduced. CA(RTD_{BW}) also had a high transmission rate (80%), but BW(RTD_{CA}) was poorly transmitted (4%) (Table 3). Using *A. gossypii* as a vector, CAwt and CAnm were readily transmitted, but, as expected, BWwt and BWnm were not transmitted at all (Table 3). Importantly, BW(RTD_{CA}) was transmitted by *A. gossypii* (22%), but no transmission event with this species was observed with CA(RTD_{BW}) (Table 3). These data indicate that transmission by *A. gossypii* requires the presence of the CABYV RTD sequence in the viral genome. Using *N. clevelandii* as a virus source, similar observations were made with both aphid species, except that the transmission efficiency of BW(RTD_{CA}) by *M. persicae* was higher (39% versus 4% from *M. perfoliata*) (Table 3).

In a second series of experiments, aphids were allowed to feed on an artificial diet containing purified virus before their transfer to *M. perfoliata* test plants. Under these conditions, BWwt and CA(RTD_{BW}) were all readily transmitted by *M. persicae* but were nontransmissible by *A. gossypii* (Table 3). CAwt and BW(RTD_{CA}), on the other hand, were transmitted by *A. gossypii* at 58% and 24%, respectively. These observations thus confirm that the specificity of *A. gossypii* for CABYV is governed by the nature of the RTD. Furthermore, the fact that differential transmission of the hybrid viruses was also observed with purified virion preparations (in which the C-

TABLE 3. Aphid transmission of recombinant viruses from infected plants or from purified viruses[e]

Mutant	No. (%) of infected plants from virus source						
	M. perfoliata			*N. clevelandii*		Purified virus[c]	
	M. persicae (20–40 aphids)[a]	*A. gossypii* (20–40 aphids)	*M. euphorbiae* (8 aphids)	*M. persicae* (20 aphids)	*A. gossypii* (20 aphids)	*M. persicae* (20 aphids)	*A. gossypii* (20 aphids)
BWwt	5/5[b] (100)	0/6 (0)	15/15 (100)	6/6 (100)	0/5 (0)	14/14 (100)	0/14 (0)
BWnm	10/14 (71)	0/15 (0)	25/25 (100)	25/26 (96)	0/20 (0)		
BW(RTD_{CA})	1/27 (4)	5/23 (22)	1/7 (14)	9/23 (39)	4/19 (21)	1/20 (5)	4/17 (24)
CAwt	13/24 (54)	16/18 (89)	7/25 (28)			5/15 (33)	7/12 (58)
CAnm	6/18 (33)	10/18 (56)	0/16 (0)				
CA(RTD_{BW})	16/20 (80)	0/20 (0)	16/17 (94)	19/25 (76)	0/24 (0)	7/7 (100)	0/3 (0)
No virus[d]	0/13 (0)	0/13 (0)	0/8 (0)	0/10 (0)	0/9 (0)	0/4 (0)	0/4 (0)

[a] After a 24-h AAP, either 8 aphids (*M. euphorbiae*) or 20 to 40 aphids (*M. persicae* and *A. gossypii*) were transferred to *M. perfoliata* for a 4-day inoculation access period.

[b] Number of plants infected after aphid transmission (determined by DAS-ELISA)/number of plants tested. In parentheses, percentages of infected plants are shown.

[c] Final concentration of purified virus in the artificial diet ranged between 70 to 130 μg/ml.

[d] Aphid feeding on noninfected plants or on virus-free diet.

[e] For the plant acquisition results, three or five independent experiments were combined. Results were from one experiment for CA(RTD_{BW}) and no virus and from two independent experiments for the other constructs with acquisition on purified virus.

TABLE 4. Electron microscopy localization of recombinant virions in the gut of *M. persicae*[a]

Mutant	Concn (μg/mL)	No. of aphids/total no. of aphids[b]					
		Posterior midgut			Hindgut		
		Lu	Cyto	BL	Lu	Cyto	BL
$BW(RTD_{CA})$	105	1/9	0/9	0/9	2/4	1/4	0/4
$BW(RTD_{CA})$	320	13/17	8/17	6/17	15/16	12/16	5/16
$CA(RTD_{BW})$	105	13/23	12/23	9/23	23/24	0/24	0/24
No virus[c]		0/6	0/6	0/6	0/7	0/7	0/7

[a] Localization in gut lumen (Lu), cytoplasm (Cyto), or basal lamina (BL) of the posterior midgut and hindgut.
[b] Number of aphids in which virions were observed/total number of aphids analyzed.
[c] Aphids directly taken from the stock colony.

terminal part of the RTD has been lost) indicates that the N-terminal portion of the CABYV RTD contains the specificity determinants. Finally, it is noteworthy that the low content of RT* observed in purified $BW(RTD_{CA})$ and $CA(RTD_{BW})$ virions (Fig. 4) does not prevent them from being transmitted by *A. gossypii* and *M. persicae*, respectively.

BWwt and CAwt are both efficiently transmitted by *M. euphorbiae* from agroinfected plants when 20 aphids were transferred from the virus source to each test plant (data not shown), but when only 8 aphids were used per test plant, a differential effect on CABYV and BWYV transmission was observed. Thus, BWwt and BWnm were transmitted with 100% efficiency under these conditions, while transmission of CAwt occurred at 28% and no transmission events were observed with CAnm (Table 3). $BW(RTD_{CA})$ behaved like CAwt (i.e., it was poorly transmitted by *M. euphorbiae* [14%]), whereas a 94% transmission rate was observed for $CA(RTD_{BW})$ (Table 3). Thus, RTD-specific discrimination between the CABYV and BWYV recombinants is not limited to *A. gossypii* but applies for another vector as well.

Microinjection of hybrid virus into the aphid hemocoel. To determine the step(s) in the transmission pathway at which *A. gossypii* discriminates between the CABYV and the BWYV RTD, purified virus (50 μg/ml) was directly microinjected into the hemocoel of *A. gossypii* nymphs so as to bypass the gut barrier. The nymphs microinjected with $CA(RTD_{BW})$ failed to transmit the virus to *M. perfoliata* (0/14 plants infected using five microinjected aphids per plant in two independent experiments), whereas nymphs similarly microinjected with CAwt transmitted the infection to 13/14 test plants. These observations suggest that $CA(RTD_{BW})$ is either unable to cross the hemocoel-ASG barrier in *A. gossypii* or unstable in the hemolymph of this aphid species.

Role of the RTD in gut tissue specificity. Previous TEM observations have shown that BWYV and CABYV have a different intestinal tissue tropism in their vector (see above). To analyze the role of the RTD in this tissue tropism, TEM was used to localize hybrid virions in ultrathin sections of *M. persicae*.

Consistent observation of $BW(RTD_{CA})$ virions required a concentration of virus of 320 μg/ml in the diet (Table 4). Under these conditions, numerous virions were observed in the lumen of both the posterior midgut (Fig. 6a) and the hindgut, as well as within the cytoplasm at both sites (Table 4). In the cytoplasm, the virions were generally enclosed in membranous

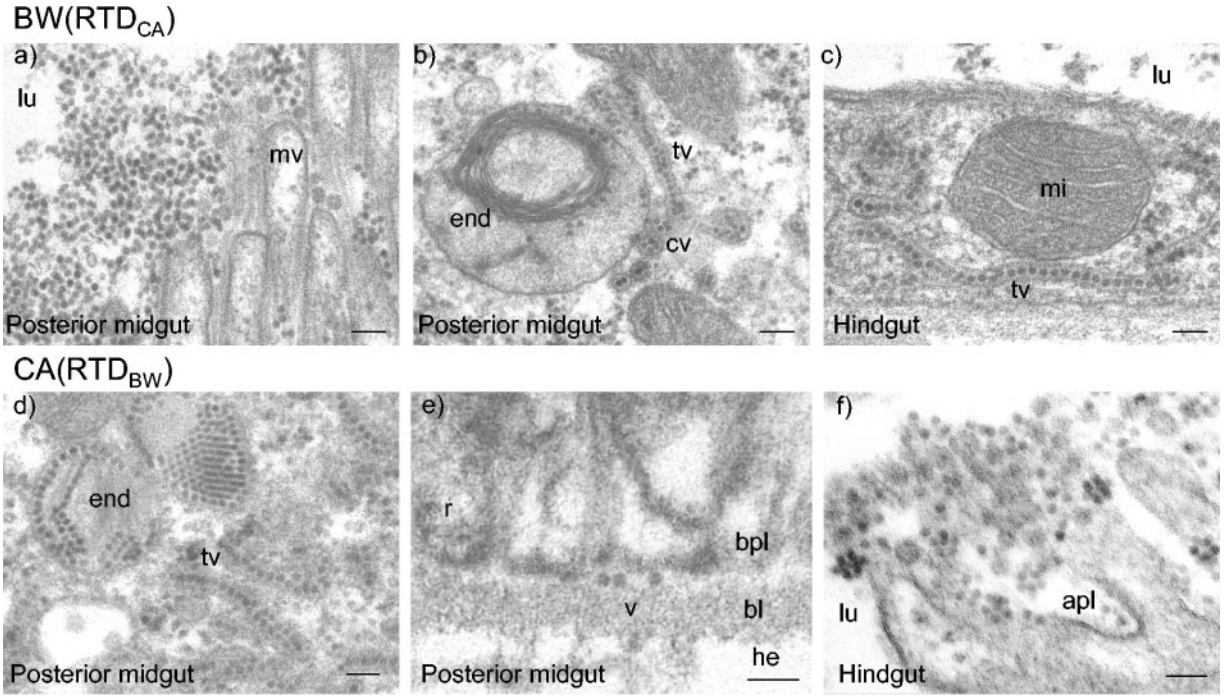

FIG. 6. Observation of recombinant virions in the gut of *M. persicae*. (a to c) $BW(RTD_{CA})$ virions in posterior midgut and hindgut cells. (d to f) $CA(RTD_{BW})$ virions in posterior midgut cells and in hindgut lumen. lu, lumen; mv, microvilli; mi, mitochondrium; tv, tubular vesicle; cv, coated vesicle; end, endodome-like vesicle; apl, apical plasmalemma; bpl, basal plasmalemma; bl, basal lamina; he, hemolymph; v, virions; r, ribosomes. Bar, 100 nm.

structures similar to those already observed in gut cells of *M. persicae* fed on BWYV or CABYV (37, 38). BW(RTD_{CA}) virions were found mainly in tubular vesicles (Fig. 6c) and occasionally in coated vesicles or endosome-like vesicles, with some of the latter also containing multilamellar material (Fig. 6b). Individual virions were also detected between the basal plasmalemma and basal lamina or embedded in the basal lamina of both posterior midgut and hindgut cells (Table 4). Thus, BW(RTD_{CA}), like CABYV, appears to be transcytosed at both locations in *M. persicae*.

The results suggesting that gut tropism of BW(RTD_{CA}) in *M. persicae* is determined by the nature of the RTD were confirmed by similar TEM studies using the reverse hybrid CA(RTD_{BW}). Virions were observed in the posterior midgut lumen, most frequently close to the apical plasmalemma microvilli, in 13 out of 23 aphids observed (Table 4). CA(RTD_{BW}) particles were observed in tubular or endosome-like vesicles in posterior midgut cells in half of the aphids examined (Table 4 and Fig. 6d). At the basal pole of these cells, isolated virus particles were occasionally observed between the basal plasmalemma and basal lamina or embedded in the basal lamina in 9 out of 23 aphids observed (Table 4 and Fig. 6e). These observations indicate that CA(RTD_{BW}) virions are released from posterior midgut cells into the hemolymph in *M. persicae*. The low number of virus particles observed at the basal pole of midgut cells is probably not due to inefficient virus movement across this barrier, since CA(RTD_{BW}) is efficiently vectored by *M. persicae* at high virus concentrations (Table 4), but rather reflects the rapidity with which virus is transported through the basal plasmalemma and basal lamina (15).

In almost all the aphids examined, free CA(RTD_{BW}) particles were abundant in the hindgut lumen close to the apical plasmalemma (Table 4 and Fig. 6f). The virions in the lumen were often present as rosette-like clusters of several particles (Fig. 6f), as previously described for CAwt virions in the lumen of its two vector species (38), suggesting that the CABYV CP may be the protein driving such aggregation. Such clusters were never observed in *M. persicae* fed on BWwt or BW(RTD_{CA}). BW(RTD_{CA}) virions were never detected inside hindgut cells or in the basal lamina surrounding the hindgut. Thus, transcytosis of recombinant CA(RTD_{BW}), like that of BWwt, appears to occur predominantly via posterior midgut cells in *M. persicae*, providing further substantiation of our hypothesis that the RTDs of BWYV and CABYV are the major determinants of gut tropism in this aphid species.

DISCUSSION

Infectivity of mutant and recombinant viruses. To address the question of poleroviruses transmission specificity, we have used a gene exchange strategy on two poleroviruses (BWYV and CABYV) that differ in their vector specificities. The virus recombinants BW(RTD_{CA}) and CA(RTD_{BW}) were competent for replication in protoplasts and for viral movement in *M. perfoliata*, a common host plant for both parental viruses. The hybrid viruses, however, showed a reduced accumulation in protoplasts and whole plants compared to the wild-type viruses. In the case of the CABYV-based viruses, this lower accumulation correlates with the 2-nt modification in the 3′ noncoding sequence introduced to create an MluI restriction site to allow the RTD sequence exchange. Thus, when the MluI site was shifted in the coding sequence of CABYV-RTD 20 or 80 nt upstream of the ORF5 stop codon, viral replication in protoplasts of the resulting mutated virus was restored to wild-type levels (data not shown). This suggests that the sequence just downstream of the ORF5 stop codon contains *cis*-acting replication signals. The 2-amino-acid change downstream of the CP stop codon, on the other hand, was neutral, since the replication rate in protoplasts of the mutated virus carrying only these changes (CAn) was similar to that of the wild-type virus.

Effect of gene exchange on host range. Agroinoculation of the recombinant viruses on CABYV- or BWYV-specific hosts revealed a complex situation. On *N. clevelandii*, a BWYV host and a CABYV nonhost, both BW(RTD_{CA}) and CA(RTD_{BW}) were infectious (Table 1). Thus, neither the origin of the RTD sequence nor the rest of the virus genome in the recombinants is a strict determinant of infectivity toward this plant species. On *C. sativus*, on the other hand, infection was obtained only with the recombinant containing the CABYV genome backbone, i.e., CA(RTD_{BW}). Thus, the primary determinant governing the ability of CABYV to infect *C. sativus* appears to reside outside the RTD, although the CABYV RTD sequence may promote the efficiency of the infection in view of the low virus titers observed in the CA(RTD_{BW})-infected plants (Table 1). In conclusion, for the two virus/host combinations studied here, there is no simple correlation between the origin of a particular sequence in a recombinant virus (e.g., the RTD) and infectivity toward a given host.

Effect of gene exchange on transmission specificity. Specific transmission, a crucial feature for all vector-borne plant viruses, is thought to result from coevolution between virus, vector, and host, and the study of the underlying mechanisms represents a challenging area of research. Even noncirculative associations display a considerable degree of specificity. For *Cucumber mosaic virus* (*Cucumovirus*, *Bromoviridae*), specific transmission by *M. persicae* or *A. gossypii* has been shown to depend on distinct amino acid motifs exclusively borne by the CP (33, 35), whereas for potyviruses (*Potyviridae*), specific motifs have been delineated on both the CP and the "helper component," and vector specificity results from specific interactions between these two proteins and the food canal epicuticle (12). Moreover, gene exchange experiments similar to ours have revealed that *Grapevine fanleaf virus* (*Nepovirus*, *Comoviridae*) transmission by the nematode *Xiphinema index* depends on the viral capsid (1). A similar role of the CP in vector specificity was demonstrated for whitefly-vectored begomoviruses (*Begomovirus*, *Geminiviridae*) (7, 22) and for *Cucumber necrosis virus* (*Tombusvirus*) transmitted by the fungus *Olpidium bornovanus* (28, 39).

Studies on many luteovirid-aphid combinations have demonstrated that efficient transport of virions across epithelial cell layers requires motifs on both the CP and the RTD (reviewed in reference 6). However, much less is known about the nature of the differential interactions that determine whether or not a particular virus can be vectored by a given aphid species. Here, we demonstrate conclusively that the specificity of transmission of BWYV and CABYV by *A. gossypii* and *M. euphorbiae* is governed by the nature of the RTD sequence. Furthermore,

our findings suggest that the critical sequences are probably located primarily in the N-terminal portion of the RTD. This is the first report of modification of vector specificity induced by genetic manipulation of a luteovirid. In recent studies on *Soybean dwarf virus*, sequence analysis of strains differentially transmitted by *Aulacorthum solani* or *Acyrthosiphon pisum* provided circumstantial evidence that specificity correlates to the N-terminal half of the RTD (19, 40, 41). These findings with a different virus-vector combination are clearly consistent with our observations and suggest that the N-terminal (conserved) half of the RTD may be the site of the primary vector specificity determinant for all luteovirids.

Effect of gene exchange on tissue tropism in the vector. Within the luteovirid-aphid associations examined so far, virus transport from gut lumen into hemocoel was found to occur through either the midgut (e.g., BWYV or *Potato leafroll virus* in *M. persicae*), the hindgut (e.g., *Barley yellow dwarf virus* and *Cereal yellow dwarf virus* in cereal aphids), or both organs (e.g., CABYV in *M. persicae* and *A. gossypii*). Our present data allow us to conclude that the RTD of CABYV mediates the acquisition across both the midgut and hindgut, whereas the RTD of BWYV is associated with only midgut acquisition. The mechanism by which BWYV and CABYV tissue tropism is determined is not understood, but it could reflect the location of virus-specific receptor-like molecules in the midgut and hindgut apical epithelia. Tissue tropism has been reported in a few other vector associations such as the propagative transmission of *Tomato spotted wilt virus* (*Tospovirus*, *Bunyaviridae*) by thrips (31, 32). For arboviruses (arthropod-borne vertebrate-infecting viruses), tissue tropism of flavivirus particles in the mosquito vector has been related to the distribution of specific receptors (29). Similarly, BWYV and CABYV might be recognized by distinct receptors, with the BWYV-specific receptor present in only the midgut and the CABYV receptor present in both midgut and hindgut. Alternatively, BWYV and CABYV might employ the same receptor in the midgut, while uptake of CABYV is mediated by a different receptor in the hindgut. In the latter case, it might be possible to selectively inhibit hindgut or midgut uptake by mutations in the RTD sequence. Finally, more complex situations can be envisaged. For example, CABYV and BWYV uptake could be mediated by a single receptor or receptor complex which is present in both the midgut and hindgut, but differences in the lumen "environment," such as pH, which is known to vary along the alimentary tract in aphids (11), could alter the affinity of the receptor (24) and/or the conformation of BWYV RTD so that it no longer binds to the hindgut-borne receptor without having such an effect on the CABYV RTD. Evidently, much remains to be learned about the specific tissue tropism of luteovirids and about luteovirid-vector interactions in general, and recombinant viruses, such as those described here, should be extremely useful in future investigations.

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